IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Cascalho et al.
Serial No.: 10/591,212
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Group No.: 1644
Examiner: Wen
Entitled: **METHODS FOR ALTERING T CELL DIVERSITY**

DECLARATION OF MARILIA I. CASCALHO, MD, PhD UNDER 37 C.F.R. § 1.131

EFS Web Filed
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Examiner Wen:

I, Marilia I. Cascalho, hereby declare and state, under penalty of perjury, that:

1. I am one of the inventors of the above-named patent application (hereinafter "present application").

2. It is my understanding that in the Office Action mailed June 15, 2010, the Examiner has cited as prior art Goronzy et al. (Arthritis Res. Ther. 2003, 5:225-234), which was published on-line on August 8, 2003. On page 5 of the Office Action, the Examiner cites Goronzy et al. as providing the suggestion for monitoring T-cell diversity:

> *However, it would have been obvious to one of ordinary skill in the art to monitor T cell diversity in view of the teaching by Goronzy et al. (see entire document). In particular, Goronzy et al. taught that T cell diversity can decrease due to clonal expansion in*

response to chronic infections such as HIV (see page 226, right column, Threats to T-cell diversity) and in autoimmune disease such as rheumatoid arthritis (see page 227, right column, T-cell diversity in rheumatoid arthritis) studies have done to monitor. Although, Goronzy et al. did not specifically teach monitoring T cell diversity, upon reading the prior art, one of ordinary skill in the art would have been reasonably expected to monitor T cell diversity in subjects with HIV infection or rheumatoid arthritis.

3. Attached at TAB A is a draft manuscript submitted to the Editors of the Journal *Immunity* that is prior to the August 8, 2003 publication date of the Goronzy et al. reference. This draft manuscript shows that the inventors of the present application described administering polyclonal immunoglobulin to a subject and monitoring T cell diversity in the subject prior to the August 8, 2003 publication date of the Goronzy et al. reference (see, page 11 2nd and 3rd paragraphs; page 15, 2nd paragraph; page 7, last paragraph; page 8, first partial paragraph; and Table 1). It is noted that the shaded boxes represent redacted dates.

4. I further declare that all statements made herein are of my own knowledge, are true, and that all statements are made on information and belief that are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application of any patent issued thereon.

Dated: 11/10/2010

Marilia I. Cascalho, MD, PhD

TAB A

REDACTED

B.J. Fowlkes, PhD
Editor, *Immunity*
1100 Massachusetts Avenue
Cambridge, MA 02138

Dear Dr. Fowlkes:

Enclosed, please find a manuscript entitled "B cell-dependent TCR diversification" for consideration for publication by *Immunity*. The manuscript reports that B cells bearing diverse B cell receptors are necessary for generation of T cell diversity in the thymus.

Diversity of T cells has been thought to result from the interaction between precursor T cells and thymic epithelial cells. Recent evidence suggests, however, that other cells might promote selection of diverse T cells, but what these "other" cells might be is not known (Martinic et al., 2003; Zinkernagel and Althage, 1999). Here we report that B cells are needed to generate wild type T cell diversity because mice that lack B cells have 0.08% TCR Vβ diversity of wild type. We also conclude that B cells' antibody diversity is necessary for the establishment of TCR Vβ diversity since mice that have oligoclonal B cells have 1.2% TCR Vβ diversity of wild type. Our report thus reveals a heretofore unrecognized function of B cells and provides insight into the mechanism by which T cell diversity is generated.

We believe that this work is appropriate for *Immunity*, since it is of general interest to scientists who study lymphocyte development, immune responses to infections such as HIV and transplantation, and immune tolerance. The concept here put forward may contribute a solution for observations of defective T cell functions in B cell immunodeficiencies and/or in situations of B cell oligoclonality.

The following scientists might be helpful as potential reviewers, since they are well-known experts in the field of lymphocyte development:

Cooper, Max D., MD
Investigator, Howard Hughes Medical Institute
University of Alabama at Birmingham
378 Wallace Tumor Institute
Birmingham, AL 35294-3300 (USA)
Phone: 205-975-7203
max.cooper@ccc.uab.edu

Sprent, Jonathan, MD, PhD
Professor, Department of Immunology, IMM4
Scripps Research Institute
10550 N Torrey Pines Road
La Jolla, CA 92037-1092 (USA)
Phone: 858-784-8619
jsprent@scripps.edu

Thank you for your consideration.

Sincerely,

Marilia Cascalho, MD, PhD
Assistant Professor of Surgery, Immunology and Pediatrics

References

Martinic, M.M., Rulicke, T., Althage, A., Odermatt, B., Hochli, M., Lamarre, A., Dumrese, T., Speiser, D.E., Kyburz, D., Hengartner, H., and Zinkernagel, R.M. (2003). Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC. Proc Natl Acad Sci USA *100*, 1861-1866.

Zinkernagel, R.M., and Althage, A. (1999). On the role of thymic epithelium vs. bone marrow-derived cells in repertoire selection of T cells. Proc Natl Acad Sci USA *96*, 8092-8097.

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B cell-dependent TCR diversification

Cristina M. João[1], Brenda M. Ogle[1,2], Carlota Gay-Rabinstein[1], Jeffrey L. Platt[1,3,4,5] and Marilia Cascalho[1,3,5]

Transplantation Biology Program[1], and the Departments of Physiology[2], Immunology[3], Surgery[4] and Pediatrics[5]
Mayo Clinic, 200 First Street SW, Medical Sciences Building 2-63, Rochester, Minnesota 55905

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Address correspondence to:
Marilia Cascalho, MD, PhD
Transplantation Biology
Mayo Clinic
200 First Street SW
Medical Sciences Building 2-63
Rochester, Minnesota 55905
Phone: (507) 538-1536
Fax: (507-284-4957
E-mail: cascalho.marilia@mayo.edu

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Summary

T cell diversity was once thought to depend on the interaction of T cell precursors with thymic epithelial cells. Recent evidence suggests, however, that diversity might arise through interaction of developing T cells with other cells, the identity of which is not known. Here we show that T cell diversity is driven by B cells and by immunoglobulin. T cell receptor Vβ diversity of splenocytes and thymocytes in mice that lack B cells is 0.08% and 0.0006% of wild type, respectively, and of splenocytes and thymocytes in mice with oligoclonal B cells is 1.2% and 0.01% of wild type, respectively. Adoptive transfer of B cells or administration of immunoglobulin increases thymocyte diversity in mice that lack B cells, 22 and 7 fold, respectively. These findings reveal a heretofore unrecognized and vital function of B cells for generation of T cell diversity and suggest a potential approach to immune reconstitution.

Introduction

The generation of T cell receptor diversity is initiated by recombination of the variable (V), diversity (D) and joining (J) gene segments and originates the variable region of the T cell receptor (TCR) genes in T cell precursors in the thymus. While V(D)J recombination generates billions of different TCRs, only a small fraction of these (5%) is expressed by the mature thymocytes. Thymocytes that fail to produce TCR or that produce TCR that fails to recognize MHC bearing self peptide are eliminated. Thymocytes bearing self-reactive TCR are also eliminated (negative selection), leaving a small fraction of thymocytes surviving (positive selection). Thus, positive and negative selection give rise to a primary T cell repertoire that recognizes self-MHC (restriction) with moderate avidity that is not self-reactive, thus establishing the diversity of naïve T cells (approximately 10^7 different T cells in humans and 10^6 in mice) (Arstila et al., 1999; Casrouge et al., 2000). Therefore, assuming an equal contribution of V(D)J recombination, the diversity of newly made thymocytes reflects the efficiency of selection.

Positive selection and T cell restriction have been thought to result from the interaction of developing T cells with thymic epithelial cells. This conclusion was deduced from experiments in which lethally irradiated recipient mice of different MHC haplotypes were reconstituted with bone marrow cells obtained from H-2^b TCR transgenic mice. In these chimeras, T cells were positively selected only when the thymic MHC was of the H-2^b haplotype, indicating that H-2^b expression by bone marrow-derived cells alone was not sufficient to promote positive selection. The conclusion that thymic epithelial cells mediate positive selection was also indicated by the work of Benoist and Mathis who showed that expression of MHC class-II on cortical thymic

epithelium was sufficient to achieve positive selection of thymocytes, whereas expression of the same MHC antigens on hematopoietic derived cells was not.

Some questioned whether cells other than thymic epithelial cells mediate positive selection. For example, Pawlowski et al. and Hugo et al. showed in separate experiments that MHC class-I or MHC class-II bearing fibroblasts injected in thymi of β_2m deficient or MHC class-II deficient mice were able to mediate positive selection. Bix and Raulet showed that bone marrow-derived cells in β_2m deficient bone marrow chimeras promoted positive selection of $CD8^+$ thymocytes. On the other hand, MHC class II-positive bone marrow-derived cells did not rescue $CD4^+$ T cells in MHC class II-deficient mice. If some experiments establish that positive selection can, in some cases, be mediated by non-thymic epithelial cells, the question of how physiologic these interactions may be is not resolved. Zinkernagel and collaborators (Martinic et al., 2003) generated tetraparental aggregation chimeras in which thymic epithelial cells expressed one MHC, T and B cells another and non-thymic epithelial cells expressed either parent's MHC. In these chimeras, T cells were restricted to the MHC expressed on the thymic epithelial cells, as expected, and to the MHC expressed by the hematopoietic cells. These results indicated that non-thymic epithelial cells promote positive selection of thymocytes, as well as thymic epithelial cells (Martinic et al., 2003). Which hematopoietic cells were responsible for the positive selection of thymocytes was not determined.

Besides the question of which cells mediate positive selection of thymocytes is the question of which cells provide the source of peptides. One speculation has been that the peptides originate in the thymic epithelium; however, the repertoire that is available form this source may not suffice. Selection of a diverse T cell repertoire requires diverse peptides presented in the context

of self MHC. Thus, analysis of T cell repertoire in mice that express MHC associated with a single peptide have a markedly constrained T cell repertoire.

Since B cells are normal constituents of the thymus (Inaba et al., 1988) and they may present peptides derived from the immunoglobulin variable regions or peptides derived from antigens expressed endogenously, we hypothesized that B cells would potentially serve as a source of peptide diversity in the thymus. In fact, B cells are one of the major cell types expressing MHC class II and are capable of presenting antigens to T cells.

We tested this hypothesis by comparing the diversity of the TCR repertoire in mice with varying B cell numbers and varying B cell diversity, and by testing whether transfer of B cells or immunoglobulin could rescue TCR diversity.

Results and Discussion

To test whether B cells might contribute to thymic selection of T cells, we compared the T cell repertoires in B cell-deficient mice (Chen et al., 1993; Kitamura et al., 1991) and mice with oligoclonal B cells, the quasi-monoclonal mouse (Cascalho et al., 1996), with the T cell repertoires in wild type mice. The B cell-deficient strains consisted of the JH-/- mice obtained by gene targeted deletion of the JH segments (Chen et al., 1993) and the μMT mice, obtained by gene targeted disruption of the μ immunoglobulin heavy chain membrane exons (Kitamura et al., 1991). The JH-/- mice lack mature B cells and immunoglobulin (Chen et al., 1993). In contrast, the μMT mice were found to produce IgA (Macpherson et al., 2001) and other non-IgM isotypes, presumably the product of a small population of mature B cells. The quasi-monoclonal mouse is

hemizygous for a rearranged VDJ segment at the Ig heavy (H) chain locus, the other allele being non-functional owing to deletion of the JH gene segments and produces only λ light chain. The QM mouse has a normal number of B cells. The primary B cell repertoire of the quasi-monoclonal mouse is monospecific and recognizes the hapten (4-hydroxy-3nitrophenyl) acetyl (NP). Somatic hypermutation and secondary rearrangements change the specificity of 20% of the peripheral B cells (Cascalho et al., 1997), originating an oligoclonal secondary B cell repertoire that is 0.01% of wild type (data not shown).

Thymocyte development is perturbed in mice that lack B cells. To test whether B cells might contribute to thymic selection, we compared thymocyte populations in JH-/-, μMT and C57BL/6 mice. Mice that lack B cells (JH-/-) had 4.3-fold fewer thymocytes than wild type (C57BL/6) mice and 3-fold fewer thymocytes than mice with a partial B cell deficiency (μMT) (**Figure 1A**). The smaller number of thymocytes in JH-/- mice is owed to a 3.9-fold decrease in the number of $CD4^+CD8^+$ thymocytes (**Figure 1A**), compared to the numbers in C57BL/6 mice.

Increased apoptosis in the thymic cortex of mice that lack B cells. The fewer $CD4^+CD8^+$ thymocytes in JH-/- mice was in part, the result of higher levels of cell death. Terminal deoxynucleotidyl transferase (TdT)-mediated UTP-biotin nick end labeling (TUNEL), which detects DNA strand breaks in cells undergoing apoptosis, revealed increased apoptosis in the thymic cortex of JH-/- mice compared to μMT or C57BL/6 mice (**Figure 1B**). Increased cell death could be the consequence of decreased positive selection and/or increased negative selection. Because defective positive selection is accompanied by cortical thymocyte apoptosis

(Surh and Sprent, 1994), our results are compatible with a role for B cells in promoting thymic positive selection.

The numbers of recent thymic emigrants are maintained, and thymocyte proliferation is increased in mice that lack B cells.

We next asked whether the numbers of recent thymic emigrants were maintained in B cell-deficient mice. The numbers of recent thymic emigrants were identified based on their tendency to take up relatively low levels of BrdU according to the method of Tough and Sprent (Tough and Sprent, 1996) in JH-/-, μMT and C57BL/6 mice fed BrdU for 14 consecutive days. **Figures 2A** and **2B** show no differences in the proportions of recent thymic emigrants in $CD4^+$ or $CD8^+$ naïve T cells analyzed in B cell-deficient (JH-/- and μMT) and B cell-sufficient mice (C57BL/6), suggesting that thymic output is maintained under the conditions of B cell deficiency and decreased thymocyte number.

Next we asked whether thymocytes proliferate to a greater extent in mice that lack B cells than in mice that have B cells in order to maintain thymic output in the presence of increased cell death. Cell cycle analysis of thymocytes of JH-/-, μMT and C57BL/6 mice revealed a 1.5 fold increase in the numbers of cycling thymocytes in JH-/- comparatively to μMT and C57BL/6 mice (Figure 2C). Our results thus suggest that lack of B cells and increased cell death leads to thymocyte proliferation, perhaps as a compensatory mechanism to maintain the thymic output.

Contraction of the T cell receptor repertoire in mice that lack B cells. If the thymus of B cell-deficient mice has fewer thymocytes owing to increased death but a normal T cell egress, one

might predict that the TCR repertoire would be contracted, owing to clonal expansion of the fewer surviving thymocytes. As a direct test of this idea, we assayed T cell receptor (TCR) diversity in JH-/-, μMT and in C57BL/6 mice. For this analysis, we measured the TCR Vβ chain diversity by a method that allows direct quantification of TCR diversity (Ogle et al., manuscript submitted). By this approach, diversity of the TCR Vβ chain in a population is proportional to the hybridization frequency (hits) of TCR Vβ chain-specific RNAs on a gene chip (Ogle et al., manuscript submitted). The actual diversity is calculated according to a standard curve obtained by hybridizing to a similar gene chip oligonucleotide mixtures of known diversity (**Figure 3A**). **Figure 3B** shows that the diversity of the TCR Vβ chain in JH-/- splenocytes was 4.8×10^2 per 10 μg of RNA and 0.08% of the TCR Vβ chain diversity measured in C57BL/6 splenocytes (6.0×10^5 per 10 μg of RNA). In contrast, the diversity of the TCR Vβ chain in μMT splenocytes (3.5×10^4 per 10 μg of RNA) was 5.8% of the diversity measured in C57BL/6 splenocytes.

Because decreased diversity of splenocytes could reflect mechanisms other than altered thymic selection, we measured TCR Vβ chain diversity in thymocytes obtained from JH-/-, μMT and C57BL/6 mice. **Figures 3C** and **3D** show that the TCR Vβ chain diversity of JH-/- thymocytes (6.5×10^2 per 10 μg of RNA) was 0.0006% of wild type (1.1×10^8 per 10 μg of RNA), while the TCR Vβ chain diversity of μMT thymocytes (4.2×10^4 per 10 μg of RNA) was 0.04% of wild type (1.1×10^8 per 10 μg of RNA). By showing profoundly decreased thymocyte diversity in B cell deficient mice, our results indicate that B cells promote thymic selection.

B cell diversity promotes T cell diversity. Selection of a diverse T cell repertoire requires T cell receptor recognition of diverse self-peptides in the context of self-MHC. Because the variable

regions of heavy and light chain of antibodies are a potential source of diverse self-peptides, we wondered whether T cell diversity depended on the diversity of the B cells. To address this question, we determined TCR Vβ chain diversity of splenocytes and thymocytes obtained from quasi-monoclonal mice that have oligoclonal B cells (Cascalho et al., 1996; Cascalho et al., 1997). Quasi-monoclonal mice produce normal numbers of B cells, 80% of which are mono-specific (Cascalho et al., 1996). V_H gene replacement and somatic hypermutation change the specificity of 20% of the B cells in the quasi-monoclonal mice (Cascalho et al., 2000). The diversity of J_H4 containing heavy chains in quasi-monoclonal mice splenocytes was only 0.01% of wild type. **Figures 3B** and **3C** show that the TCR Vβ chain diversity of quasi-monoclonal splenocytes and thymocytes was 7.6×10^3 and 1.3×10^4 per 10 μg of RNA, corresponding to 1.3% and 0.01% of the TCR Vβ chain diversity of wild type (6.0×10^5 and 1.1×10^8 per 10 μg of RNA), respectively.

The reduced T cell diversity in quasi-monoclonal mice indicates that if diverse B cells promote diversification of thymocytes, oligoclonal B cells do not.

Mature B cells are absent from JH-/- and μMT thymi. Our results imply that B cells promote thymic selection of a diverse T cell repertoire. However, in addition to mature B cells, B cell precursors are also present in the thymus. Thus, B cell precursors in addition to mature B cells could contribute to selection of T cells. **Figure 4A** shows that JH-/- and μMT thymi had fewer $CD19^+$ cells in the thymic cortex and lacked $CD19^+$or $B220^+$ cells in the medulla in comparison to C57BL/6 mice. In contrast, the numbers of $B220^+$ cells were comparable in the thymic cortices of JH-/-, μMT and C57BL/6 mice. To discriminate B cell precursors from mature B cells, we analyzed thymocytes of JH-/-, μMT, QM and C57BL/6 mice by flow cytometry.

Figure 4B shows that mature B cells (IgM^+ and $B220^+$) are missing from JH-/- and μMT thymi in comparison to QM and C57BL/6 thymi. There were no significant differences in the proportion of pre-B ($IgM^-CD43^-B220^+$) (Li et al., 1993) and pro-B ($IgM^-CD43^+B220^+$) cells (Li et al., 1993) present in JH-/-, μMT, QM or C57BL/6 thymi (**Figure 4 C**). Notably, pre-B cells were nearly absent in the thymi of mice of all genotypes while present in the bone marrow (**Figure 4C**) in accord with the results of Hashimoto et al. Our findings thus suggest that mature B cells rather than B cell precursors promote thymocyte selection and the generation of T cell-diversity.

Delayed rejection of H-Y discordant grafts by JH-/- mice. T cell function is thought to depend, in part, on the number of T cells bearing each antigen-specific TCR that, in turn, reflects T cell diversity. We therefore asked whether the functions of T cells differ in mice with a contracted T cell repertoire (JH-/- and μMT) from the functions of T cells in mice with wild type T cell diversity (C57BL/6). To this effect, we compared the kinetics of rejection of male skin grafts by female recipients in JH-/-mice compared to μMT and to C57BL/6 mice. The rejection of skin grafts from male mice by female recipients depends on T cell recognition of male minor histocompatibility (H-Y) derived peptides presented in the context of MHC class I and class II molecules (Scott et al., 1995; Silvers et al., 1968). Rejection of H-Y discordant grafts is a more sensitive test of T cell function than rejection of MHC incompatible grafts because the frequency of antigen specific T cells is ~10^4 fold less frequent in the former than in the latter (Kanagawa et al., 1982; Suchin et al., 2001).

Figure 5 shows that JH-/- female mice rejected male skin grafts in 26 ± 5.4 days, whereas C57BL/6 female mice rejected male skin grafts in 15 ± 1.7 days ($p = 0.002$, t test). μMT mice rejected male skin grafts in 17 ± 3.3 days, which is in accord with the findings of Fuchs and colleagues (Fuchs and Matzinger, 1992) and a result not significantly different from C57BL/6 mice. These results indicate that a substantial reduction of diversity (5.8% of wild type in μMT mice) is compatible with wild type rejection kinetics.

T cell diversity and rejection of H-Y incompatible grafts in JH-/- mice reconstituted by adoptive transfer of B cells or immunoglobulin.

Next we tested whether B cells and/or immunoglobulin could increase TCR diversity in mice that lack B cells by adoptive transfer. To this end, we injected newborn JH-/- mice with bone marrow-derived B cells obtained from adult wild type mice or immunoglobulin (mouse polyclonal IgG) and measured TCR diversity 5 weeks later.

Table I shows that injection of bone marrow-derived B cells in newborn JH-/- mice increases splenocyte and thymocyte TCR Vβ diversity 82- and 22-fold, respectively, considering the median value in each group. On the other hand, injection of polyclonal IgG in newborn JH-/- mice increases TCR Vβ diversity in splenocytes and thymocytes by 1434- and 7-fold, respectively. These results thus suggest that one mechanism by which B cells promote TCR Vβ diversity is through the production of immunoglobulin. Since injection of immunoglobulin alone increases TCR diversity, we wondered whether adoptively transferred B cells produced serum immunoglobulin. To find out, we quantified serum immunoglobulin by an ELISA assay (Cascalho et al., 1996). **Figure 6** shows that JH-/- mice had no measurable serum

immunoglobulin; μMT mice, in contrast, had measurable serum immunoglobulin that is 4.8% the concentration of C57BL/6 mice. JH-/- mice with adoptively transferred B cells had no measurable serum immunoglobulin, while JH-/- mice injected with polyclonal immunoglobulin had an average of 769 μg/ml of serum immunoglobulin. Because both immunoglobulin injections and B cell transfer in the absence of serum immunoglobulin increased T cell diversity in mice that lack B cells, our results indicate that serum immunoglobulin is a mechanism that helps to generate TCR diversity, but that B cells also contribute to T cell diversity independently of serum immunoglobulin.

B cell-reconstituted mice rejected male grafts in 21.7 ± 11 days (n=4), which is no different from wild type recipients. Our results indicate that reconstitution of mice that lack B cells at birth with B cells purified from bone marrow led to re-establishment of properties of T cells. On the other hand, immunoglobulin-treated mice showed delayed graft rejection (34.4 ± 18 days (n=5)), suggesting that IgG injections did not re-establish the functional properties of T cells.

Here we report that B cells drive the selection of a diverse repertoire of thymocytes and peripheral T cells, thus overturning the longstanding notion that T and B cells develop independently (Szenberg and Warner, 1962). Generation of a diverse TCR repertoire relies on the quasi-random recombination of the TCR variable region gene segments and on positive and negative selection (Goldrath and Bevan, 1999). Positive selection and MHC restriction are generally thought to depend on interaction of T cell precursors with thymic epithelial cells (Gill et al., 2002); however, this concept is at odds with experimental evidence indicating that mice bearing T cells and thymic epithelial cells that are haplo-incompatible are also restricted to the

MHC of non-thymic epithelial cells (Martinic et al., 2003; Zinkernagel and Althage, 1999; Zinkernagel et al., 1980). These observations could suggest that B cells colonizing the thymus promote positive selection and restriction of T cells. Our results showing reduced TCR diversity in mice that lack B cells (JH-/-) or have very few B cells (μMT) could be explained if B cells promoted positive selection of thymocytes. In agreement with the concept that B cells promote positive selection is the finding of a decreased number of $CD4^+CD8^+$ thymocytes and increased cell death in the thymic cortex of JH-/- mice. This concept is in accord with the findings of Suhr and Sprent (Surh and Sprent, 1994). We cannot, however, rule out the possibility that B cells decrease negative selection of thymocytes. However, some reports suggest that thymic B cells promote, rather than decrease, negative selection of T cells to murine mammary tumor virus (MMTV) and minor lymphocyte stimulating (*mls*) antigens and to I-E expressed only on B cells.

If B cells promote TCR diversity, how can we reconcile our results showing that the B cell-deficient μMT mice produce a TCR repertoire that is 4.5-fold more diverse than the TCR repertoire of quasi-monoclonal mice that have normal numbers of B cells? We considered the possibility that diversity of B cells may be more important than the number of B cells. Since selection of a diverse T cell repertoire in the thymus is thought to depend, at least in part, on the diversity of peptides presented by self-MHC, we hypothesized that B cells may provide an alternative source of diverse peptides derived from the variable region of immunoglobulin, or from captured peripheral antigens. We tested this hypothesis in mice with quasi-monoclonal B cells that are one thousand-fold less diverse than wild type B cells and that have normal numbers of T cells. Our findings of severely contracted T cell diversity in quasi-monoclonal mice support the concept that B cell receptor diversity is critical to the selection of diverse T cells. Thus, while the thymic epithelial cells may produce some of the peptides required for positive

selection, these may not be diverse enough to assure survival of thymocytes representing wild type TCR diversity.

Since μMT mice produce serum immunoglobulin of all isotypes with the exception of IgM and have a TCR repertoire that in the thymus is 64-fold more diverse than the TCR repertoire of JH-/- mice, we wondered whether secreted immunoglobulin could promote TCR diversification independently of B cells. Our results showing increased diversity of T cells following injection of polyclonal IgG indicate that some of the effects of B cells in the selection of T cells can be mediated by secreted immunoglobulin. However, adoptive transfer of B cells also increases TCR diversity in JH-/- mice in the absence of detectable serum immunoglobulin. Thus, we conclude that B cells and immunoglobulin promote TCR diversification independently.

Our findings of B cell-dependent TCR diversification reveal a heretofore unrecognized and vital function of B cells that may help to explain the phenotype of B cell-deficient human subjects and suggest a potential approach to immune reconstitution.

Experimental Procedures

Mice. JH-/-, μMT and quasi-monoclonal mice were previously described. C57BL/6 and μMT mice were purchased from the Jackson Laboratories. All mice were between 6 and 16 weeks of age and housed in a specific pathogen-free facility at the Mayo Clinic. All animal experiments were carried out in accordance with protocols approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Adoptive transfer. Bone marrow cells were harvested from C57BL/6 mice, and lymphocytes were isolated by Ficoll-paque gradient (Amersham Biosciences, Piscatanaway, New Jersey). 1 x 10^7 B cells purified with a Miltenyi Biotec isolation kit were injected *i.p.* in newborn mice. The injected cells contained 1% of $CD3^+CD4^+$; 2% of $CD3^+CD8^+$ cells and 76% of $CD19^+$ cells.

Immunoglobulin reconstitution. JH-/- mice were injected *i.p* weekly with 250 μg of mouse polyclonal immunoglobulin (Seroteca) since birth. Serum levels of total immunoglobulin were tested at 5 weeks after birth.

FACS analysis. Thymocytes were obtained by mincing thymi through a 0.70 μm mesh followed by red blood cell hemolysis in a standard NH_4Cl lysis buffer (Cascalho et al., 1996). Total thymocyte numbers were counted with a Coulter counter. Cells were stained with one, two or three of the following monoclonal antibodies (all the antibodies were from BD Pharmingen) as described (Cascalho et al., 1996). Fluorescein isothyocyanate (FITC)-conjugated: rat anti-mouse CD4 (GK 1.5), rat anti-mouse CD43 (Ly-48, leukosialin) and mouse anti-BrdU antibodies; phycoerythrin (PE)-conjugated: rat anti-mouse CD8α (53-6.7), rat anti-mouse CD19 (1D3), rat anti-mouse IgM^b (Igh-6b)(AF6-78); and biotin-conjugated: rat anti mouse B220 (16A), rat anti-mouse CD62L (LECAM-1, Ly22) and rat anti-mouse CD3ε (145-2C11). Lymphocytes were gated on the light scatter plot by backgating onto $CD4^+CD3^+$and $CD8^+$ $CD3^+$ cells; numbers of the thymocyte sub-populations were determined by multiplying the percentage as defined by gating on the FACS plot, by their total number.

DNA analysis. Thymocytes (10^6/ml) were washed with ice-cold PBS and fixed in 70% ethanol at -20°C for at least 2 h. After fixation, cells were washed twice with PBS and incubated in 50 µl of DNA extraction buffer (0.2 M phosphate citrate buffer, pH = 7.8) at 37°C for 30 min in the shaker. Following DNA extraction, the cells were stained with propidium iodide in a solution containing 10 ml of 0.1% (v/v) of Triton X-100 in PBS, 200 µl of 1 mg/ml PI (Molecular Probes) and 2 mg of DNase-free Rnase A (Sigma), for 30 min at room temperature. Detection of propidium iodide fluorescence was read at red wavelength in a FACScan flow cytometry (Becton Dickinson) and analyzed with the ModFit *LT* software.

Immunohistological analysis. Thymi removed from six to eight week old mice were oriented and covered with O.C.T (Sakura, Torrance, CA), snap-frozen by pre-cooled isopentane and stored at -85°C. Four micron thick frozen sections were mounted on positively-charged microscope slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and stored at -85°C. Before processing, sections were air-dried at room temperature, fixed 10 minutes in 4°C acetone, air-dried for an additional 10 minutes, then post-fixed for two minutes in 100 mM Tris-buffered 1% paraformaldehyde containing 1mM EDTA, pH 7.2, and rinsed with phosphate buffered saline (PBS, pH=7.2). Prior to staining, the specimens were incubated in 0.3% hydrogen peroxide in 0.1% sodium azide (aq) solution to quench the presence of endogenous peroxidase. Specimens were incubated with rat monoclonal antibodies to murine CD19 (clone 1D3, BD Pharmingen, San Diego, CA) and to CD45R/B220 (clone RA3-6B2, BD Pharmingen), rinsed with PBS, then detected by mouse serum preabsorbed, affinity purified, biotinylated Goat IgG anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), followed by PBS rinses and the tertiary application of horseradish-conjugated streptavidin (Dako, Carpinteria, CA). Slides were

developed by incubation with a peroxidase substrate NovaRED (Vector Laboratories, Burlingame, CA), which resulted in an insoluble reddish-brown precipitate, followed by counterstaining with a progressive alum-hematoxylin, dehydrated in graded ethanols, cleared in xylene changes, and coverslipped with Cytoseal-Xyl (Stephens Scientific, Kalamazoo, MI). Digital images were obtained using a brightfield microscope equipped with a CCD digital camera (SPOT II, Diagnostic Instruments, Sterling Heights, MI).

TUNEL. Apoptotic cells were detected in cryostat sections of thymi by *in situ* terminal deoxynucleotidyltransferase-mediated 2'- deoxyuridine 5'-triphosphate nick end-labeling (TUNEL), performed according to the manufacturers instructions (ApopTag ™plus Peroxidase kit, Serologicals Corporation).

Determination of TCRVβ diversity.

Isolation of RNA. Spleens or thymi harvested from mice were placed in RPMI and pushed through a 70 μm cell strainer. Lymphocytes were isolated by Ficoll-paque (Amersham Biosciences, Piscatanaway, New Jersey) gradient. Total RNA was obtained with Qiagen RNeasy kit (Qiagen, Inc., Valencia, California) per the manufacturer's instructions. Generation of lymphocyte receptor-specific cRNA. First strand cDNA was obtained by reverse transcription with a mouse TCR Cβ reverse primer: *T7+Cβ* (5'-

GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGCTTGGGTGGA

GTCACATTTCTC-3') or with a mouse J_H4 reverse primer: *T7+CJ$_H$4* (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGGAGGAGACGGTGACTGAGGTTCCTTG-3'). Second strand synthesis and preparation of cRNA was done according to affimetrix standard protocols (Affymetrix, Inc., Santa Clara, California).

Application of cRNA to the gene chip. Equal amounts of cRNA from different samples were hybridized to U95B gene chips (Affymetrix, Inc., Santa Clara, California). Oligomers were biotinylated and hybridized to gene chips (U95 B human genome chips - Affymetrix, Inc., Santa Clara, CA) according to Affymetrix protocols. Gene chips were processed at the Microarray Core Facility, Mayo Clinic, Rochester, MN.

Data analysis. For each gene chip experiment, we obtained raw data corresponding to oligo location and hybridization intensity. Data were arranged in order of ascending hybridization intensity. The number of oligo locations with intensity above background (i.e., number of hits) was summed. The standard curve was generated (from hybridization of samples with known numbers of different oligos). The standard oligonucleotide mixtures were 18-mer oligomeres synthesized to obtain mixtures containing 1, 10^3, 10^6 and 10^9 different oligonucleotides. Diversity of the test samples was extrapolated from the standard curve.

Skin grafts. Skin grafts (tail to back) were performed according to standard procedures (Rosenberg, 1991). Rejection was defined by shedding of 90% or more of the graft from the graft bed. Adoptively transferred mice were grafted 3 weeks following transfer.

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Competing interests statement

The authors declare that they have no competing financial interests.

Figure Legends

Figure 1. Thymocyte numbers and thymocyte apoptosis in C57BL/6, μMT and JH-/- mice

A) Thymocyte numbers. The numbers of thymocytes corresponding to each sub-population were calculated by multiplying the respective percentage of the total events as defined in the flow cytometry dot plot analysis, by the total number of WBC obtained by counting on a Coulter. The average number of thymocytes was $1.3 \times 10^8 \pm 5.1 \times 10^7$ in C57BL/6, $8.9 \times 10^7 \pm 6.4 \times 10^7$ in μMT and $3.1 \times 10^7 \pm 1.7 \times 10^7$ in JH-/- mice. The average number of $CD4^+CD8^+$ thymocytes was $9 \times 10^7 \pm 4.4 \times 10^7$ in C57BL/6, $7.3 \times 10^7 \pm 4.7 \times 10^7$ in μMT and $2.3 \times 10^7 \pm 2.7 \times 10^7$ in JH-/- mice; the average number of $CD4^-CD8^-$ thymocytes was $5.5 \times 10^6 \pm 2.1 \times 10^6$ in C57BL/6, $3 \times 10^6 \pm 2 \times 10^6$ in μMT and $2.2 \times 10^6 \pm 2.7 \times 10^6$ in JH-/- mice; the average number of $CD4^+CD8^-$ thymocytes was $1.9 \times 10^7 \pm 2.8 \times 10^7$ in C57BL/6, $4.9 \times 10^6 \pm 2.8 \times 10^6$ in μMT and $3.5 \times 10^6 \pm 3 \times 10^6$ in JH-/- mice; and the average number of $CD4^-CD8^+$ thymocytes was $1.2 \times 10^7 \pm 1.2 \times 10^7$ in C57BL/6, $7.7 \times 10^6 \pm 1.3 \times 10^7$ in μMT and $1.5 \times 10^6 \pm 1.4 \times 10^6$ in JH-/-mice. Numbers were obtained from 8 C57BL/6, 8 μMT and 7 JH-/- mice that were between 6 and 16 weeks of age and compared for statistical significance by *t* test.

B) Apoptosis in the thymus. Apoptotic cells are stained brown and were detected in cryostat sections of thymi by *in situ* terminal deoxynucleotidyltransferase-mediated 2'- deoxyuridine 5'-triphosphate nick end-labeling (TUNEL). Photographs are representative of four different mice per genotype analyzed.

Figure 2. Recent thymic emigrants and DNA content analysis of thymocytes.

A) and B). The plots represent BrdU incorporation by splenic ($CD4^+CD62L^-$) A; or ($CD8^+CD62L^+$) B, naïve T cells. *x-axis*, CD62L staining fluorescence intensity; *y-axis*, BrdU staining fluorescence intensity. The recent thymic emigrants are the naïve $CD4^+$ or $CD8^+$ lymphocytes that incorporate low levels of BrdU (Sprent and Tough, 1994). The gates defining the recent thymic emigrants were set in dot plots analyzing BrdU incorporation by $CD4^+CD62L^+$ and $CD8^+CD62L^+$ (naïve) T cells in thymectomized mice. Since in the absence of a thymus there are no thymic emigrants, BrdU incorporation is due only to peripheral proliferation of T cells. The absence of cells with low levels of BrdU staining in these animals thus defines the recent thymic emigrants gate. Thymic output did not differ in C57BL/6, μMT and JH-/- mice. The dot plots shown are representative of 3 independent experiments with C57BL/6 and JH-/- mice and 2 with μMT mice.

C) DNA content of thymocytes from C57BL/6, μMT and JH-/- mice. Histograms of one representative experiment of a total of 4, per genotype, are shown. *x-axis*, DNA content; *y-axis*, number of cells. Diagrams depict the peaks representing the number of cells in G1, S and G2/M phase of the cell cycle. The fraction of cells in S+G2/M of the cell cycle is noted. 20,000 events were collected for each analysis.

Figure 3. TCR Vβ diversity.

A) Standard curves. *x-axis*, natural log of the number of different oligonucleotides present in the standard; *y-axis*, gene chip hybridization hits (locations with intensity above background). Each curve represents an independent experiment. The standard oligonucleotide mixtures were

18-mer oligomeres synthesized to obtain mixtures containing 1, 10^3, 10^6 and 10^9 different oligonucleotides. Oligomeres were biotinylated and hybridized to gene chips (U95 B human genome chips - Affymetrix, Inc., Santa Clara, CA) according to Affymetrix protocols. Gene chips were processed at the Microarray Core Facility, Mayo Clinic, Rochester, MN.

B) TCR Vβ diversity of splenocytes obtained from C57BL/6, μMT and JH-/- mice. *x-axis*, type of mice strain; *y-axis*, TCR Vβ diversity. Hybridization hits were summed, and the total number was used to calculate TCR Vβ diversity by comparison with known diversity oligomer mixtures (standards) run at the same time. Splenocytes were obtained from 10 to 12 week old mice, as described (Cascalho et al., 1996), and mononuclear cells isolated on Ficoll-Paque gradient (Sigma, St. Louis, MO). Total RNA was obtained with Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA). First strand cDNA was synthesized with a reverse primer containing a T7 polymerase promoter 3' overhang that annealed to the TCR β constant region, with SuperScript II Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA). Second strand was generated by nick translation. The double strand cDNA was processed for gene chip hybridization according to Affymetrix protocols. Each open circle represents one experiment, and the values indicate the mean of TCR Vβ diversity for each strain of mice. Statistical analysis was performed on each of the log transformed numeric values by t test.

C) TCR Vβ diversity of thymocytes obtained from C57BL/6, μMT and JH-/- mice. *x-axis*, mice strains; *y-axis*, TCR Vβ diversity. Thymocytes were prepared as described above from mice that were 12 to 20 weeks old. Each open circle represents one experiment, and the values indicate the mean of TCR Vβ diversity for each strain of mice. Statistical analysis was performed on each of the log transformed numeric values by t test.

D) Gene chip hybridization hits obtained from samples derived from C57BL/6 and JH-/- thymocytes. Scan of gene chips illustrating the number hits of the samples obtained from C57BL/6 and JH-/-. The bright spots correspond to the hybridization hits that correlate with TCR Vβ diversity.

Figure 4. Immunohistochemical and flow cytometric analysis of thymic B cells.

A) Immunohistochemical staining of thymic sections of C57BL/6, μMT and JH-/-. Positive cells are stained brown. JH-/- and μMT thymi had fewer $CD19^+$ cells in the thymic cortex and lacked $CD19^+$ or $B220^+$ cells in the medulla in comparison to C57BL/6 mice. In contrast, the numbers of $B220^+$ cells were comparable in the thymic cortices of JH-/-, μMT and C57BL/6 mice.

B) Flow cytometric analysis of mature B cells in the thymi of C57BL/6, QM, μMT and JH-/- mice. *x-axis*, B220 staining fluorescence intensity; *y-axis*, IgM staining fluorescence intensity. The dot plot diagrams identify mature thymic B cells that are $B220^+$ and IgM^+. Mature B cells are missing from the thymi of μMT and JH-/- but present in the thymi of C57BL/6 and QM mice. Percentages represent the proportion of thymocytes that are mature B cells. Results shown are representative of 3 mice per genotype that were between 6 and 10 weeks of age.

C) Flow cytometric analysis of pro- and pre-B cells in the thymi or bone marrow of C57BL/6, QM, μMT and JH-/- mice. *x-axis*, B220 staining fluorescence intensity; *y-axis*, CD43 staining fluorescence intensity. The plots represent IgM^- cells. Upper diagrams represent thymocytes; lower diagrams, bone marrow cells. There were no significant differences in the proportions of pre-B ($IgM^-CD43^-B220^+$) and pro-B cells ($IgM^-CD43^+B220^+$) in JH-/-, μMT, QM or C57BL/6 thymi. The percentages refer to the fraction of IgM^- cells that corresponds to the indicated

phenotype. Pre-B cells were nearly absent in the thymi of mice of all genotypes while present in the bone marrow.

Figure 5. H-Y incompatible skin grafts survival. Skin grafts (tail to back) were performed according to standard procedures. *x-axis*, days following surgery; *y-axis*, skin graft survival fraction. Rejection was defined by shedding of 90% or more of the graft from the graft bed. The average time of rejection was 15 d ± 1.7 d in C57BL/6; 17 d ± 3.3 d in the μMT and 26 d ± 5.4 d in JH-/- mice. Each group included 5 mice. Statistical analysis was done with *t* test.

Figure 6. Concentration of serum immunoglobulin in C57BL/6, QM, μMT and JH-/- mice reconstituted or not with B cells or IgG. *x-axis* represents the mice genotypes, *y-axis* represents the concentration total immunoglobulin in μg/ml.

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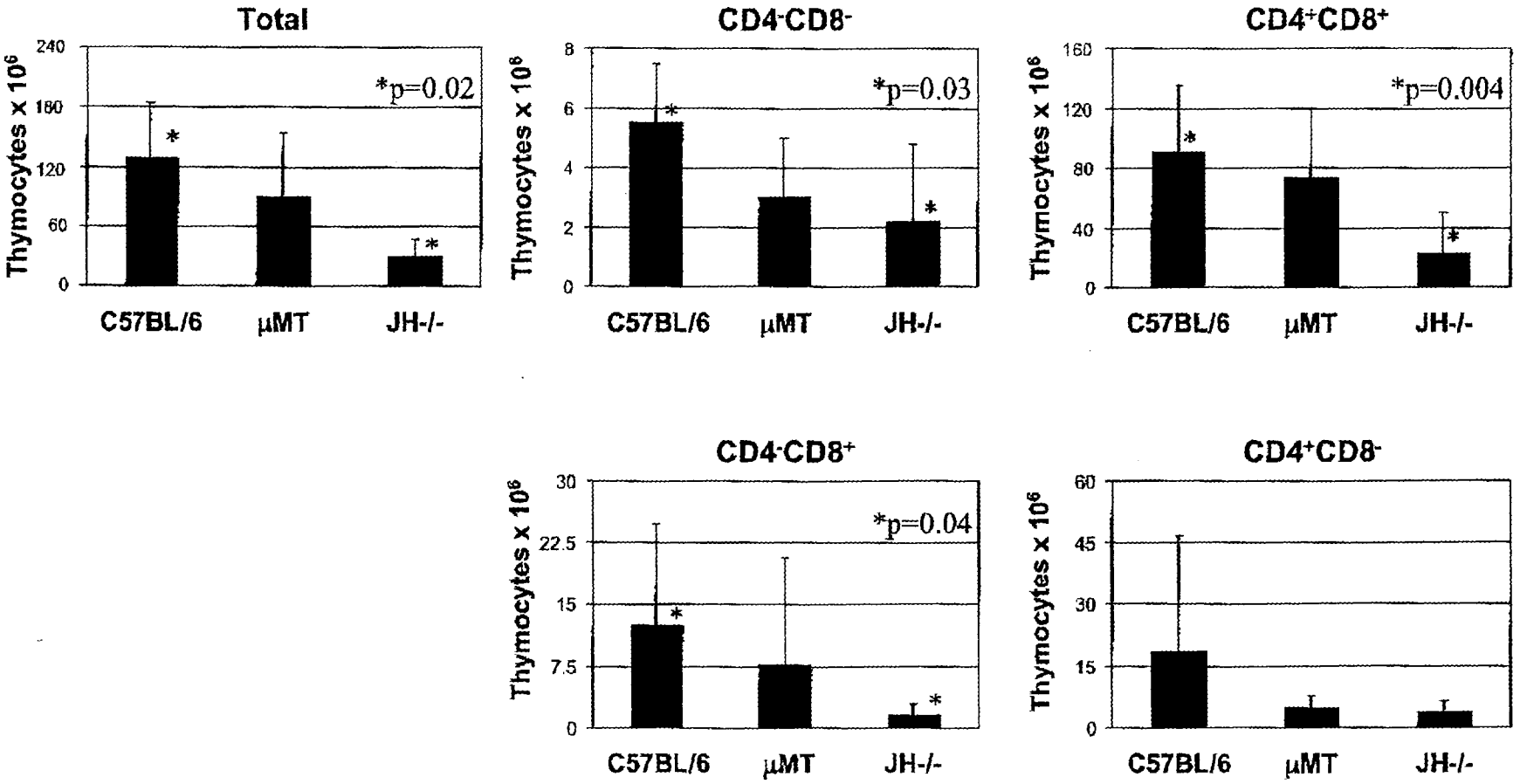
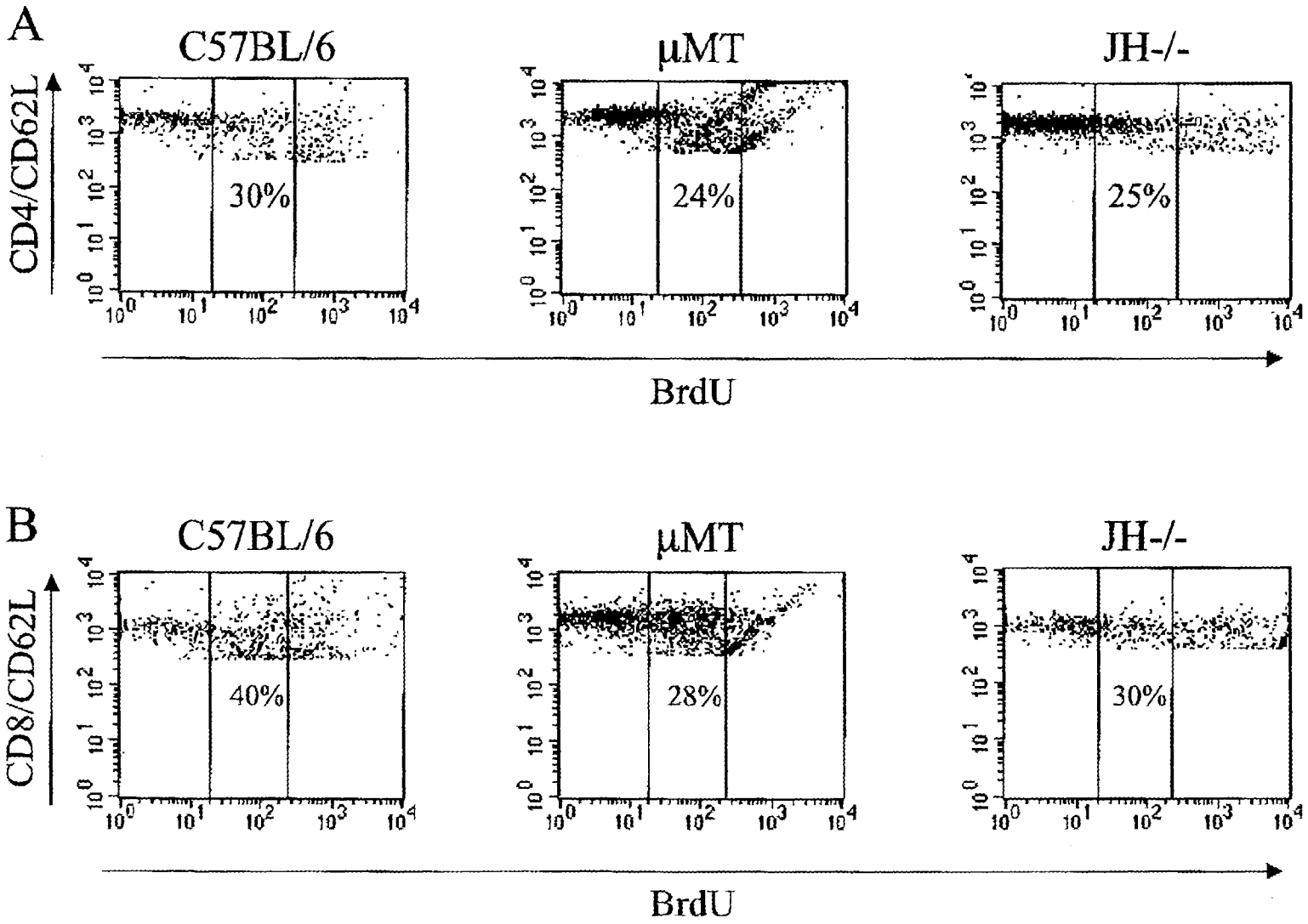
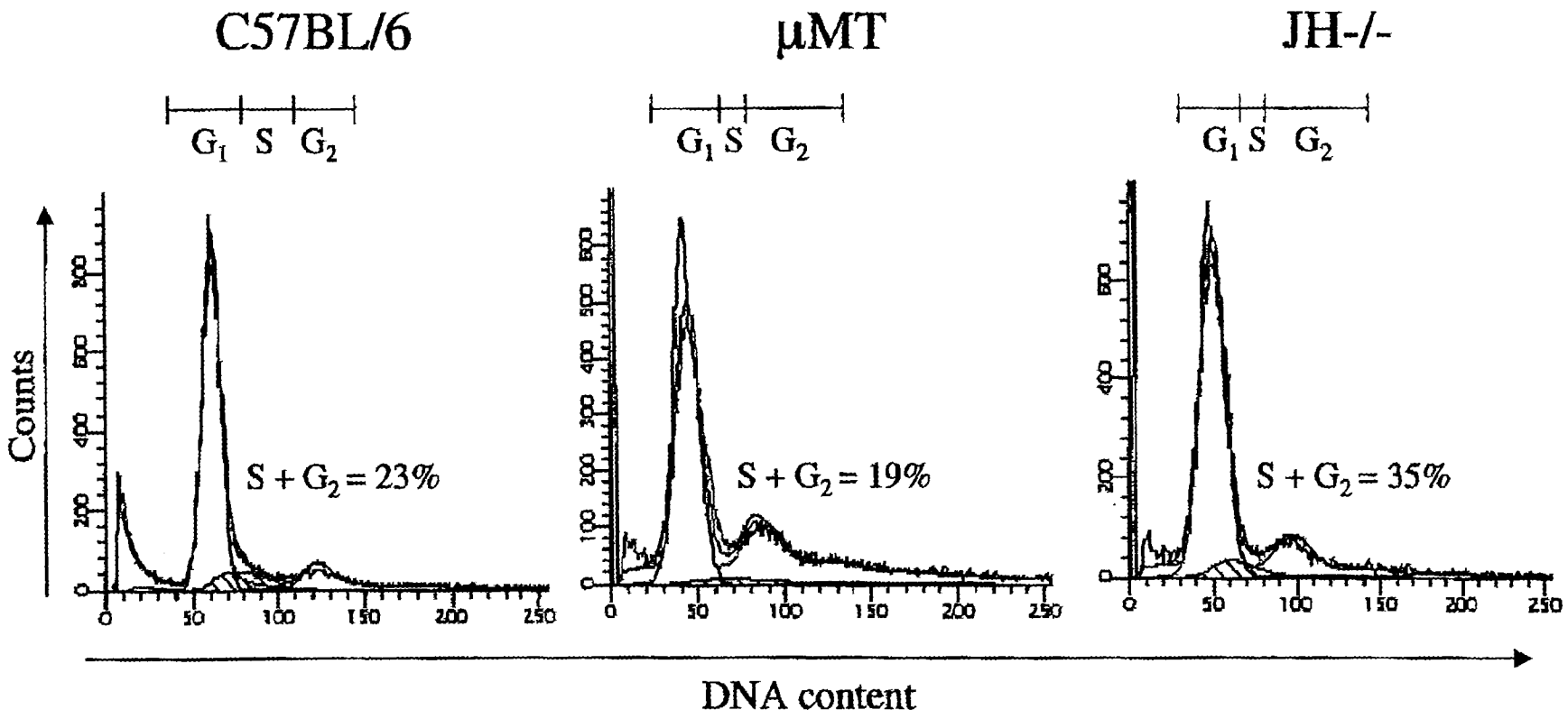
Table I. TCR Vβ diversity in JH-/- mice reconstituted with B cells or IgG

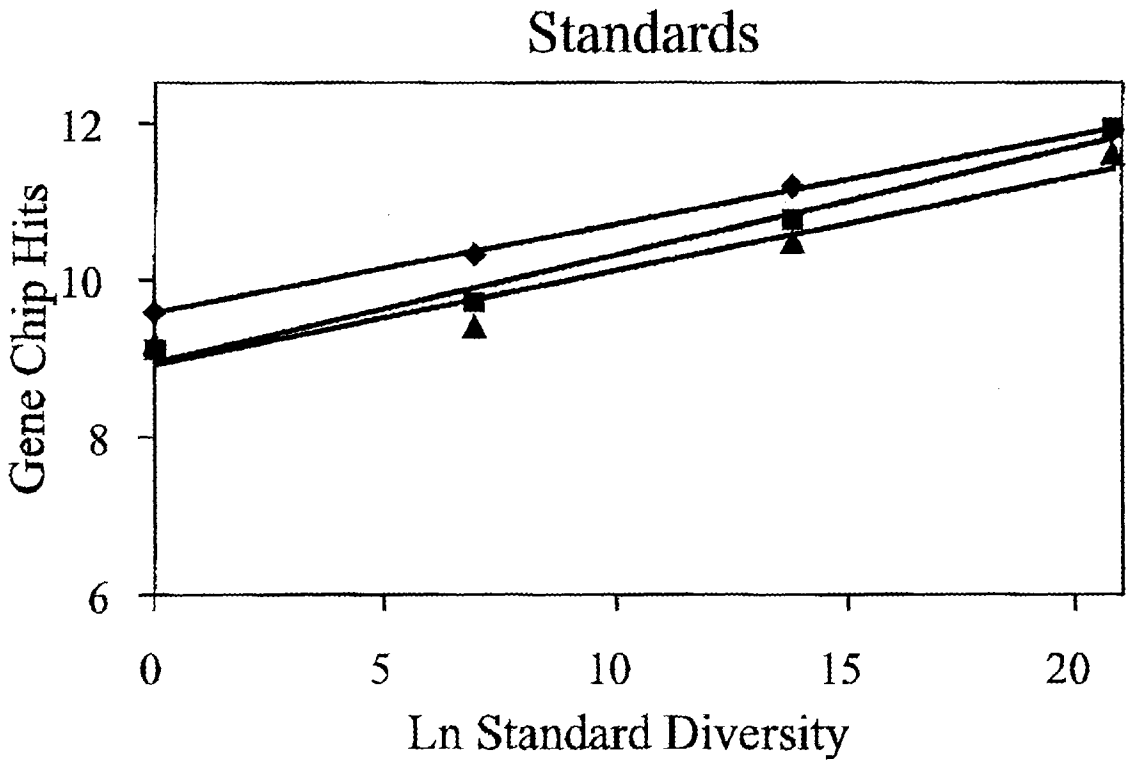
	Thymus		Spleen	
	TCR Vβ Diversity (median)	Fold Increase	TCR Vβ Diversity (median)	Fold Increase
JH-/- reconstituted with B cells	14545	22	7537	82
JH-/- reconstituted with IgG	4273	7	132204	1437
JH-/- not reconstituted	589		92	

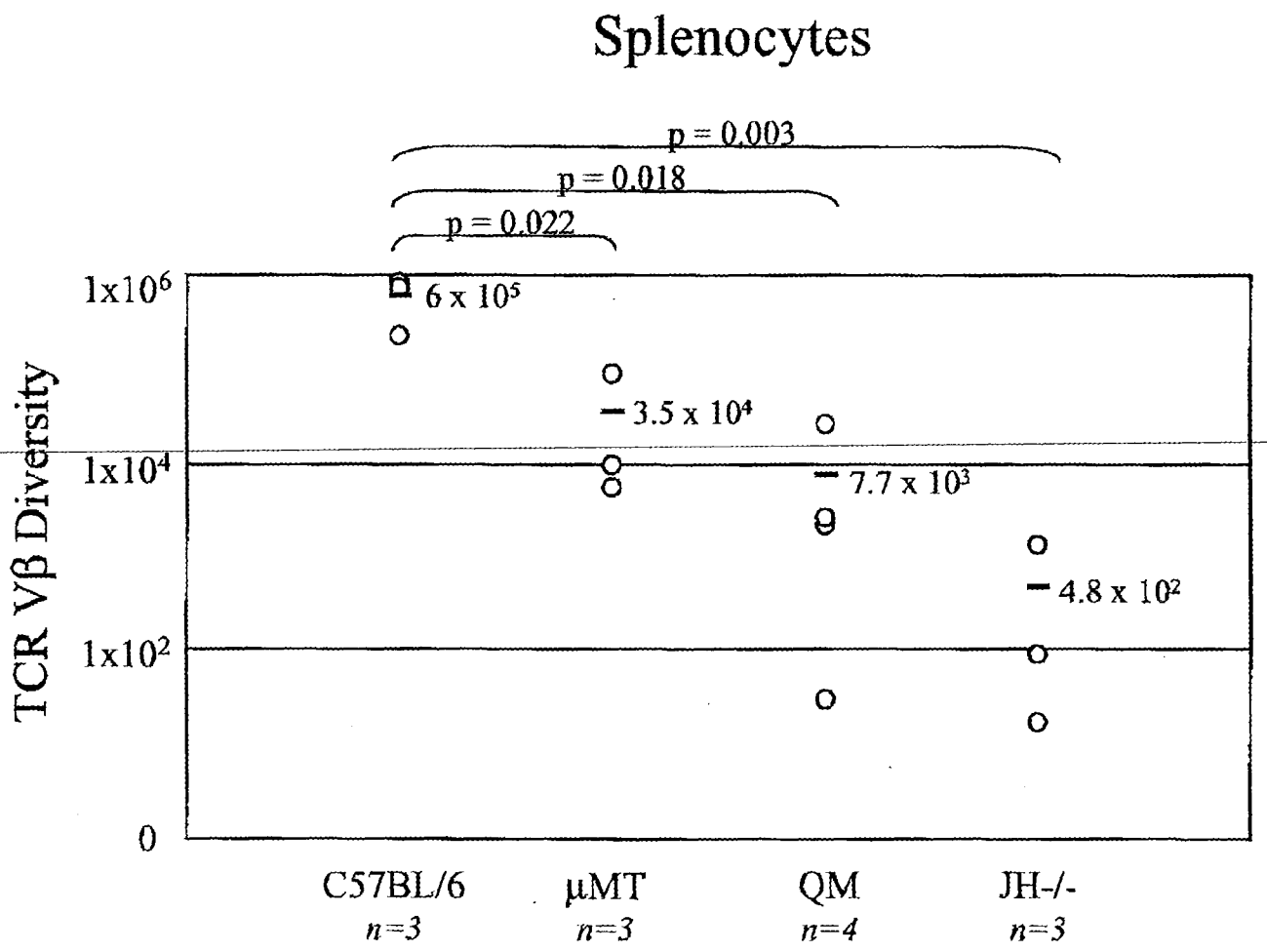

Total
Thymocytes x 10⁶
240
180
120
60
0
*p=0.02
*
*
C57BL/6
µMT
JH-/-
CD4-CD8-
Thymocytes x 10⁶
8
6
4
2
0
*p=0.03
*
*
C57BL/6
µMT
JH-/-
CD4+CD8+
Thymocytes x 10⁶
160
120
80
40
0
*p=0.004
*
*
C57BL/6
µMT
JH-/-
CD4-CD8+
Thymocytes x 10⁶
30
22.5
15
7.5
0
*p=0.04
*
*
C57BL/6
µMT
JH-/-
CD4+CD8-
Thymocytes x 10⁶
60
45
30
15
0
C57BL/6
µMT
JH-/-

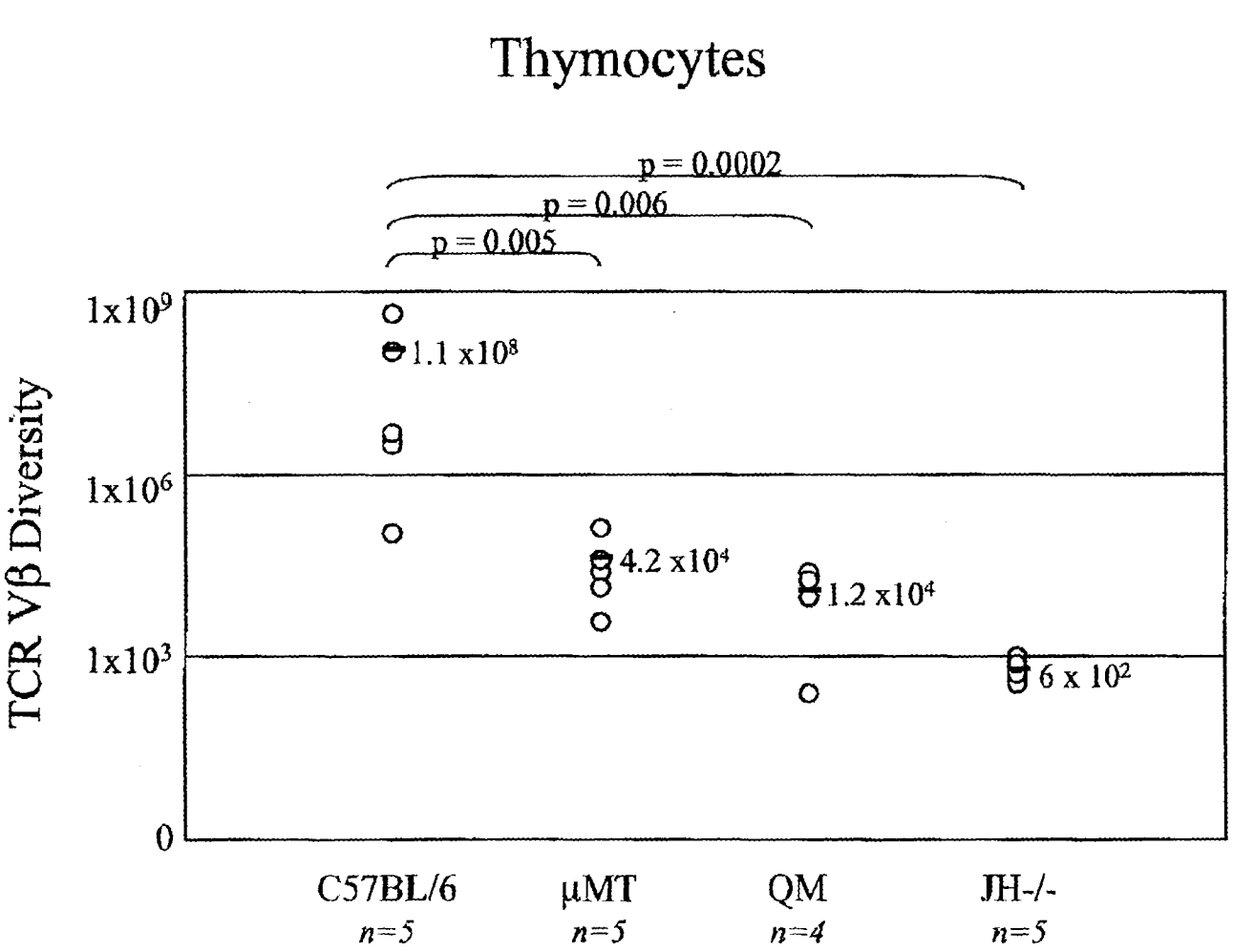
C57BL/6
μMT
JH-/-


A
C57BL/6
μMT
JH-/-
CD4/CD62L
30%
24%
25%
BrdU
B
C57BL/6
μMT
JH-/-
CD8/CD62L
40%
28%
30%
BrdU


C57BL/6
μMT
JH-/-
G1 S G2
G1 S G2
G1 S G2
Counts
S + G2 = 23%
S + G2 = 19%
S + G2 = 35%
0 50 100 150 200 250
DNA content

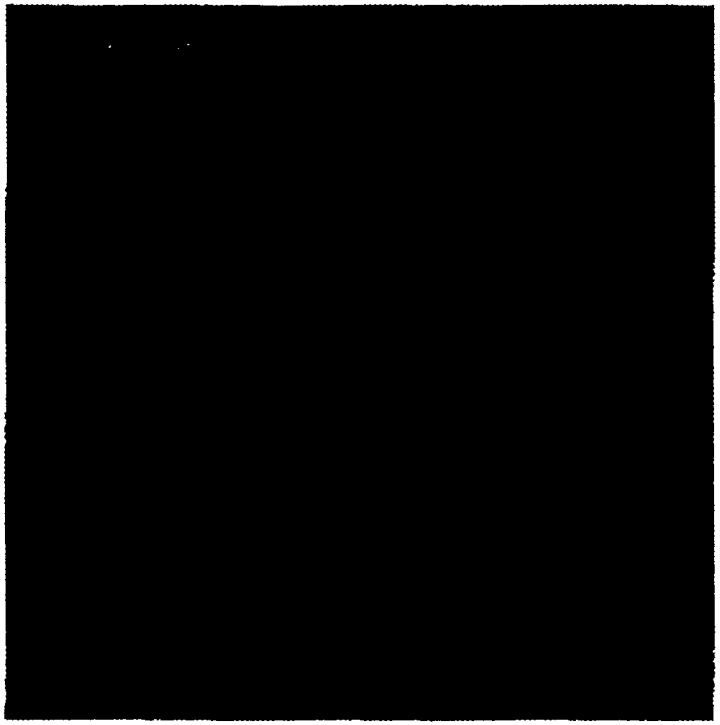

Standards
Gene Chip Hits
12
10
8
6
0
5
10
15
20
Ln Standard Diversity


Splenocytes
p = 0.003
p = 0.018
p = 0.022
1x10^6
1x10^4
1x10^2
0
TCR Vβ Diversity
6 x 10^5
3.5 x 10^4
7.7 x 10^3
4.8 x 10^2
C57BL/6
n=3
μMT
n=3
QM
n=4
JH-/-
n=3

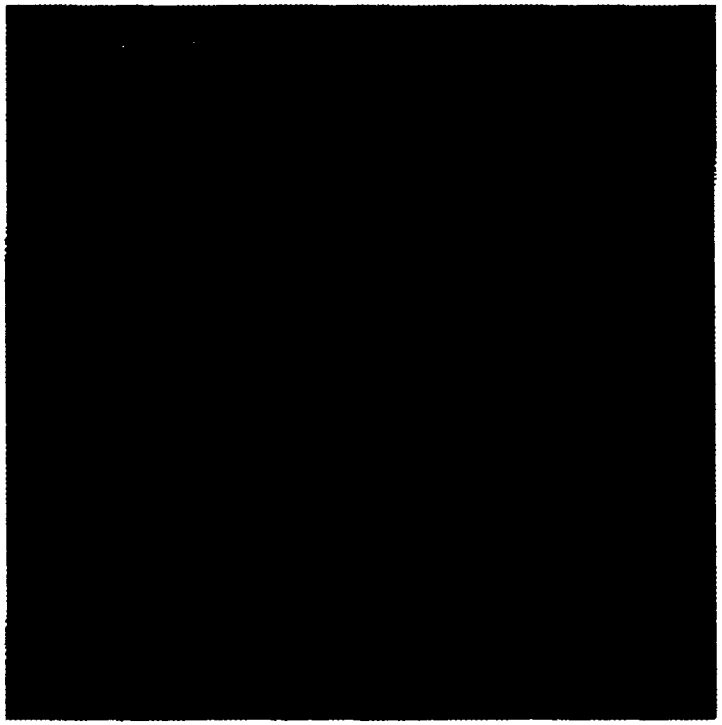

Thymocytes
p = 0.0002
p = 0.006
p = 0.005
TCR Vβ Diversity
1x10^9
1x10^6
1x10^3
0
1.1 x10^8
4.2 x10^4
1.2 x10^4
6 x 10^2
C57BL/6
n=5
μMT
n=5
QM
n=4
JH-/-
n=5

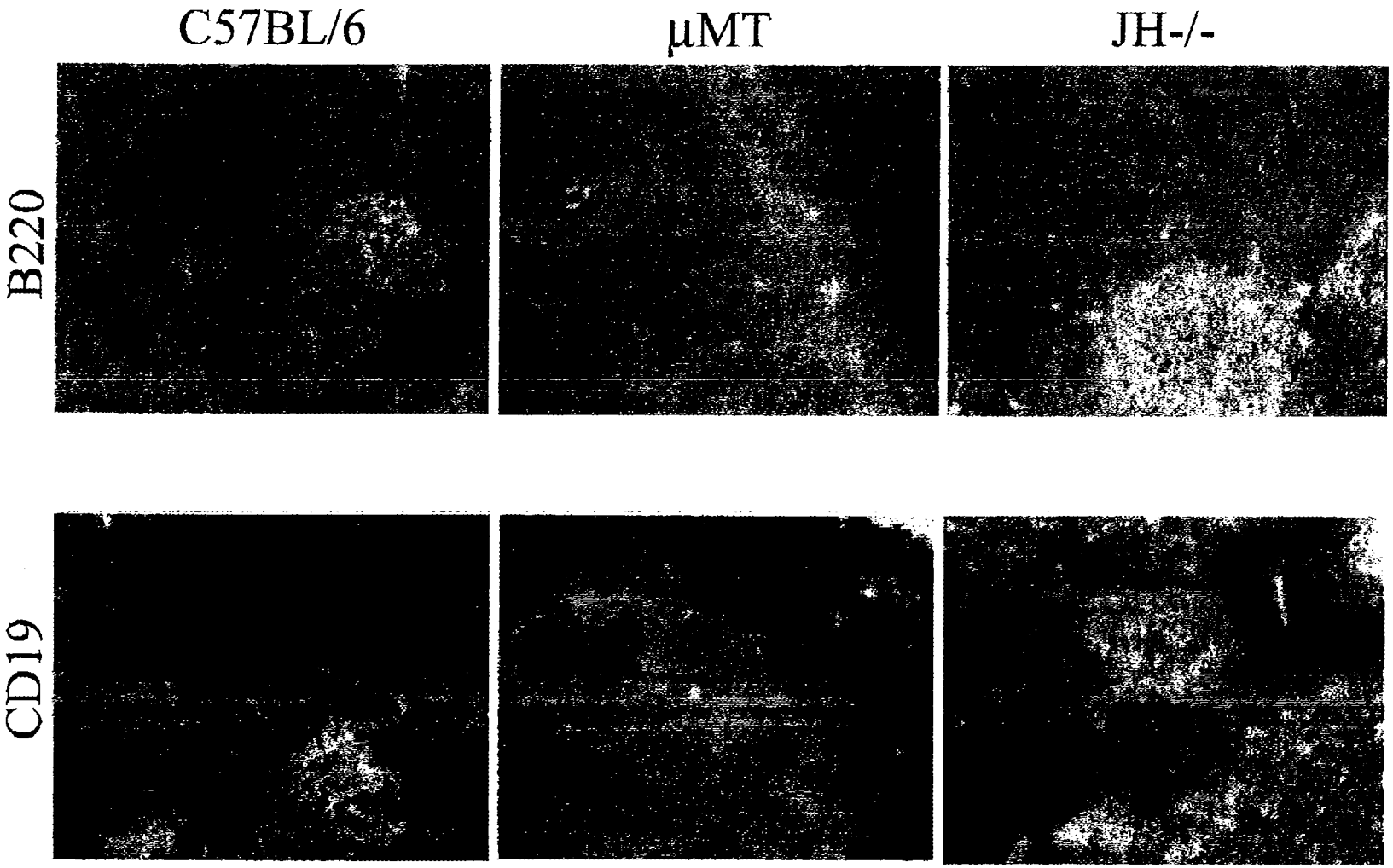
Thymocytes

C57BL/6

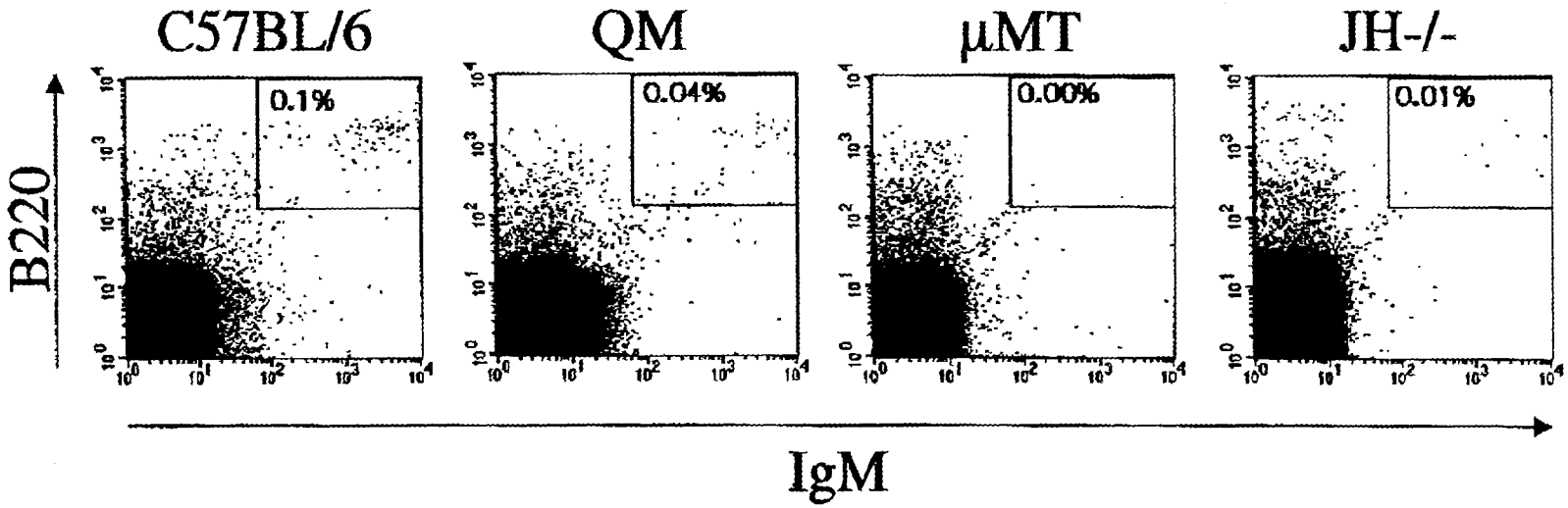


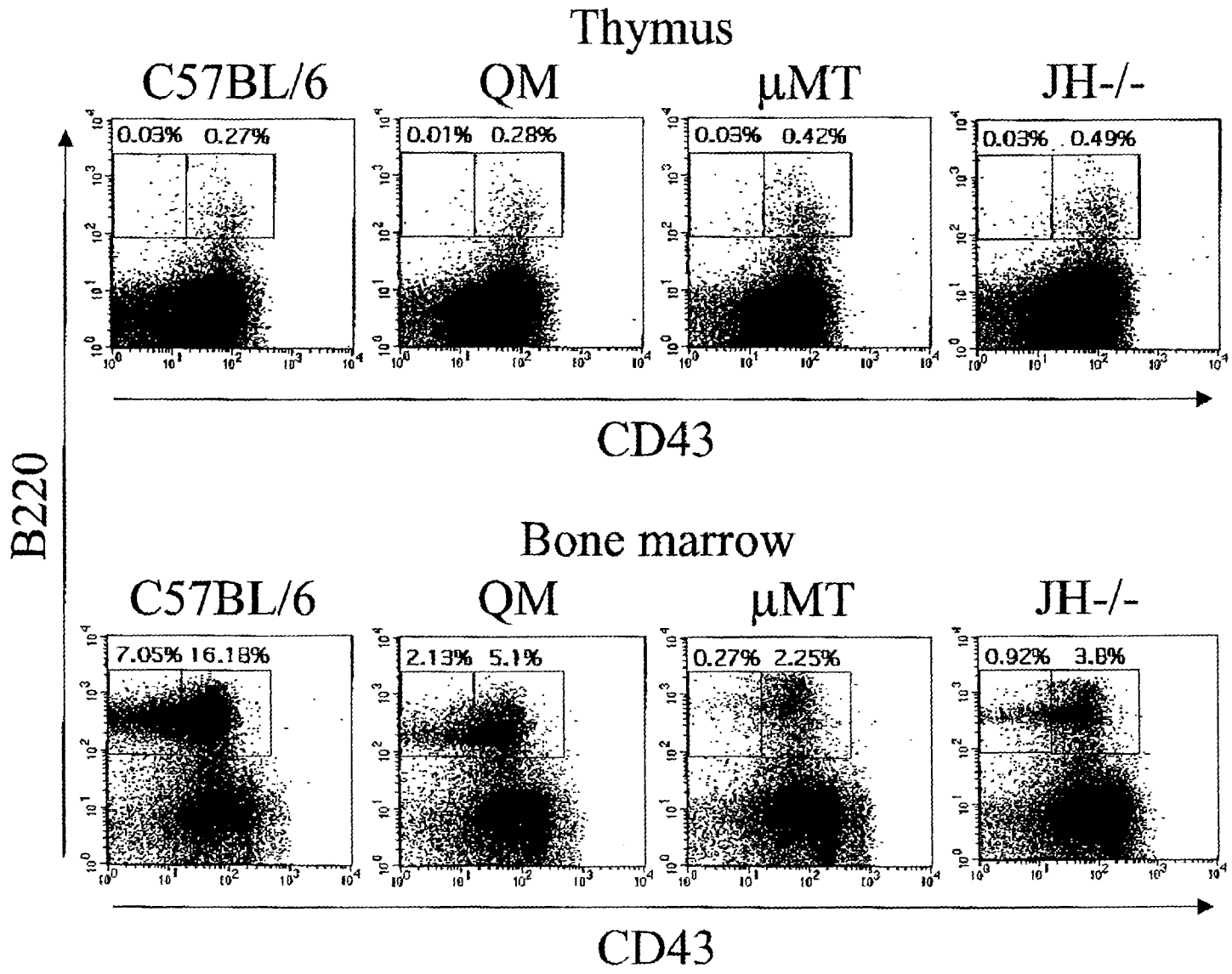
JH-/-

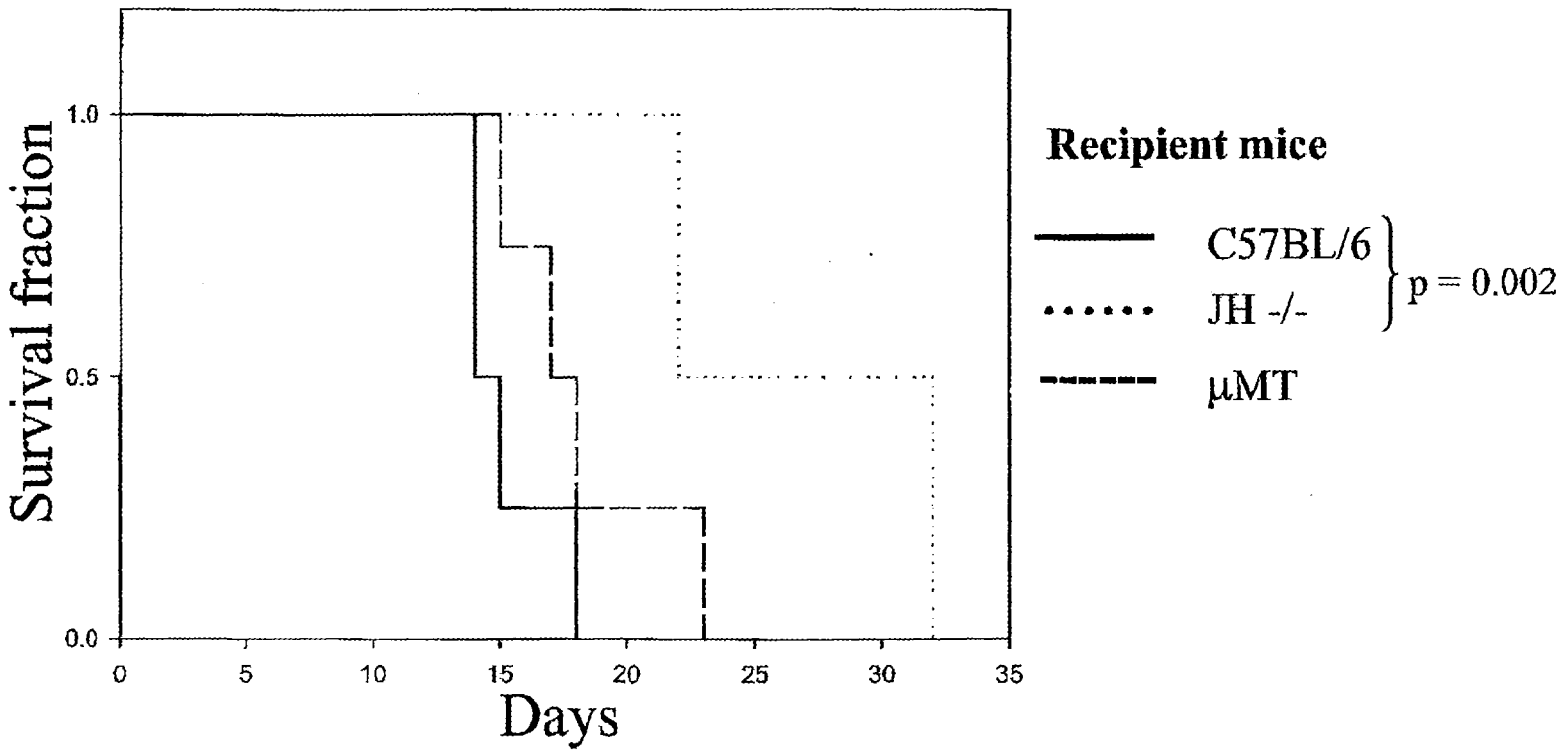



C57BL/6
µMT
JH-/-
B220
CD19

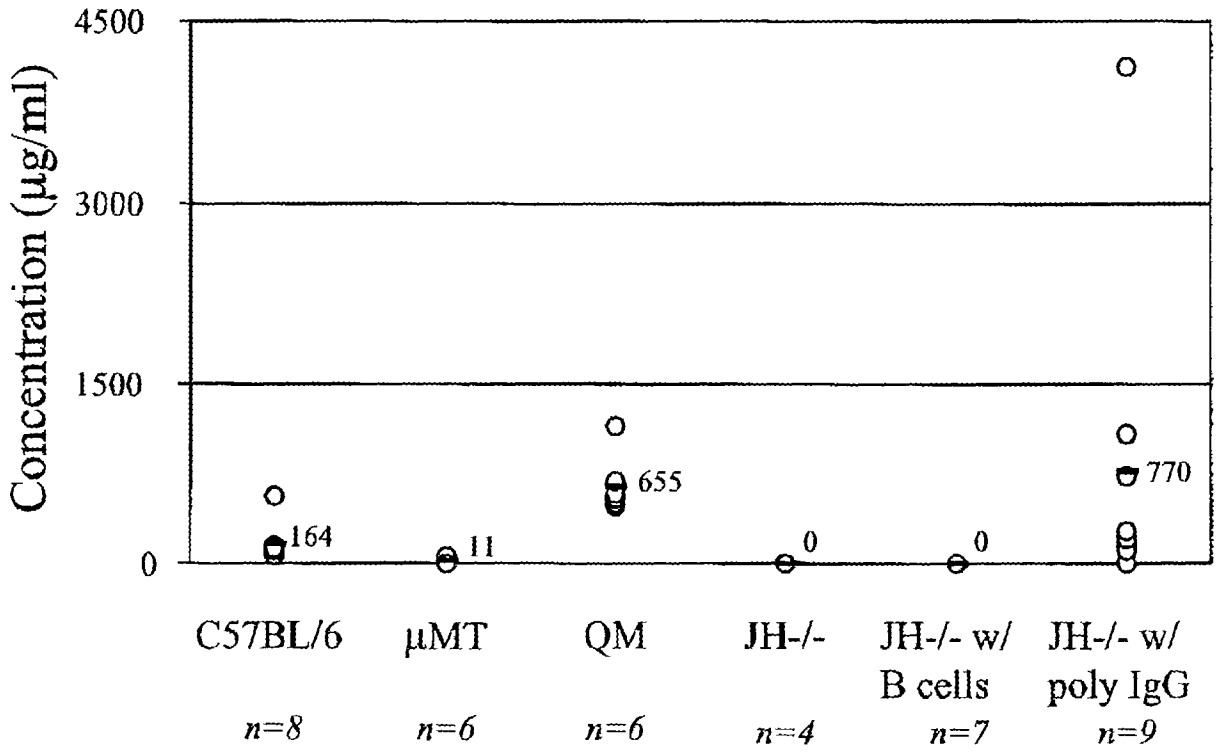
Thymus




IgM-negative cells
Thymus
C57BL/6
QM
μMT
JH-/-
0.03% 0.27%
0.01% 0.28%
0.03% 0.42%
0.03% 0.49%
B220
CD43
Bone marrow
C57BL/6
QM
μMT
JH-/-
7.05% 16.18%
2.13% 5.1%
0.27% 2.25%
0.92% 3.8%
CD43


Survival fraction
1.0
0.5
0.0
0
5
10
15
20
25
30
35
Days
Recipient mice
C57BL/6
JH -/-
μMT
p = 0.002

Total Ig


Concentration (µg/ml)
4500
3000
1500
0
164
11
655
0
0
770
C57BL/6
µMT
QM
JH-/-
JH-/- w/ B cells
JH-/- w/ poly IgG
n=8
n=6
n=6
n=4
n=7
n=9